

The *Bradyrhizobium japonicum* *phoB* gene is required for phosphate-limited growth but not for symbiotic nitrogen fixation

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Abstract

We identified by cloning and DNA sequence analysis the phosphate regulatory gene *phoB* of *Bradyrhizobium japonicum*. The deduced gene product displayed pronounced similarity to the PhoB protein of *Sinorhizobium meliloti* (71.4% identical amino acids), *Escherichia coli* (50.2%) and other bacterial species. Insertion of a kanamycin resistance cassette into *phoB* led to impaired growth of the *B. japonicum* mutant in media containing approximately 25 μ M phosphate or less. A standard plant infection test using wild-type and *phoB*-defective *B. japonicum* strains showed that the *phoB* mutation had no effect on the symbiotic properties of *B. japonicum* with its soybean host plant. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

Phosphorus in the environment is one of several important nutritional factors for the growth of bacteria. Symbiotically living bacteria such as *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, are particularly affected because the symbiotic dinitrogen fixation process is highly phosphorus-dependent [1]. Israel [1] showed that the environmental phosphorus concentration specifically influences both nodule size and nodule number in addition to being critical for supporting host plant growth.

The role of phosphorus in bacterial growth has

been investigated mainly with *Escherichia coli* (for reviews see [2,3]). The genetic control system is based on the phosphate regulatory protein PhoB. Its activity is modulated by specific phosphorylation and dephosphorylation, mediated by the transmembrane sensor protein PhoR in response to the environmental phosphate concentration. Under conditions of phosphate limitation, phosphorylated PhoB acts as a transcriptional activator by binding to a specific 18-bp sequence (*pho* box) in the promoter region of genes belonging to the so-called *pho* regulon. The *pho* regulon in *E. coli* consists of at least 31 genes arranged in eight transcriptional units, whose gene products are involved in the transport (e.g. the PstSCAB phosphate transport system) or mobilisation (e.g. the PhoA phosphatase) of phosphates and phosphorus compounds.

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Comparatively little is known about the phosphate regulatory system in symbiotically living bacteria. Only recently, Bardin et al. [4] described an ABC-type phosphate uptake system, PhoCDET, of *Sinorhizobium meliloti*, whose transcription is induced in response to phosphate starvation. An analysis of the corresponding promoter region revealed two elements that are similar to the previously described *pho* boxes present in phosphate-regulated *E. coli* promoters. Mutants having mutations in the *S. meliloti* *phoCDET* operon grew poorly at a relatively high phosphate concentration of 2 mM and failed to form nitrogen-fixing root nodules. By analogy with the situation in *E. coli* it seems reasonable to propose that the product of the *phoB* gene, which has been identified in *S. meliloti*, regulates the cellular response to environmental phosphate limitation.

We report here that the sequence analysis of a fortuitously cloned *B. japonicum* DNA region revealed an open reading frame with similarity to the phosphate regulatory gene *phoB*. The results from a mutational analysis of the *S. meliloti* phosphate uptake system PhoCDET [4] prompted us to investigate the significance of *phoB* for cell growth under phosphate limitation and for the symbiotic interaction of *B. japonicum* with its soybean host plant.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

E. coli cells were grown in Luria-Bertani (LB) medium [5] supplemented with ampicillin (200 µg ml⁻¹), kanamycin (30 µg ml⁻¹), or tetracycline (10 µg ml⁻¹), if required. The growth temperature for *E. coli* strains was 37°C. *B. japonicum* was routinely grown aerobically at 28°C in PSY medium [6]. For phosphate limitation experiments *B. japonicum* strains were propagated aerobically at 28°C in HM minimal medium [7] containing different concentrations of phosphate. Both *B. japonicum* media were supplemented with 0.1% (w/v) L-arabinose. If appropriate, antibiotics were added at the following concentrations (µg ml⁻¹): chloramphenicol, 20 (for counterselection against *E. coli* donor strains); kanamycin, 100; spectinomycin, 100; and tetracycline, 50. The spectinomycin-resistant *B. japonicum* strain

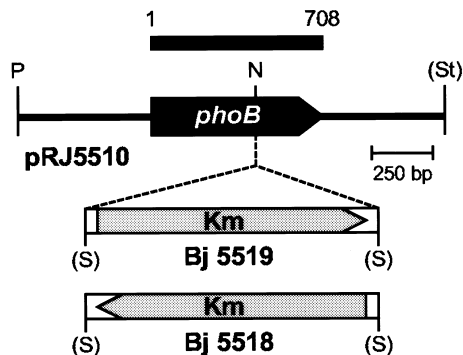


Fig. 1. Physical map of the *B. japonicum* *phoB* gene. The genetic organisation of the 1.8-kb insert of plasmid pRJ5510 and the *B. japonicum* *phoB* insertion mutations in strains 5518 and 5519 are shown. Abbreviations: Km, neomycin phosphotransferase II gene; N, *NruI*; P, *PstI*; S, *SmaI*; St, *StuI*. Restriction sites in parentheses were destroyed during the cloning procedures. The nucleotide sequence, which has been deposited in the GenEMBL data base under accession number AJ223073, and the corresponding numbers representing the first and last nucleotide of the start and stop codon of *phoB*, respectively, are indicated above the physical map.

110*spc4* [6] was used as parental strain. Strains 5518 and 5519 are derivatives of strain 110*spc4* in which a neomycin phosphotransferase II cassette (Km^r) was inserted into the *NruI* site located in the *phoB* gene. The orientation of the cassette relative to *phoB* in the individual mutants is shown in Fig. 1. Plasmid pRJ5510 is a pUC18 derivative which carries the *B. japonicum* *phoB* gene region on a 1.8-kb insert as shown in Fig. 1.

2.2. DNA manipulations

Recombinant DNA techniques were used according to standard protocols [8]. DNA was sequenced by the chain termination method [9] with a model 373 DNA sequencer and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA region sequenced and the deduced amino acid sequence were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, WI, USA; version 8.0) or the NCBI (National Center for Biotechnology Information) BLAST network server. The 'CODONPREFERENCE' program was applied using a previously established *B. japonicum* codon usage table [10].

2.3. Construction of *B. japonicum phoB* mutant strains

The 1.2-kb *Sma*I fragment of pBSL15 [11] containing the neomycin phosphotransferase II cassette (Km^r) was inserted into the *Nru*I site located in the *phoB* gene present on plasmid pRJ5510 (Fig. 1). Both orientations of the resistance cassette relative to the *phoB* gene were obtained. The inserts of the resulting plasmids were subcloned into pSUP202po13 (H.M. Fischer, unpublished) and mobilised from *E. coli* S17-1 into *B. japonicum* 110*spe*4 for marker replacement mutagenesis as described previously [12]. The correct genomic structure of these mutant strains was confirmed by Southern blot hybridisation of genomic DNA digested with appropriate restriction enzymes, using a *B. japonicum phoB* probe.

2.4. Plant infection test

The symbiotic phenotype of the *B. japonicum phoB* mutants was determined in a soybean plant infection test as described previously [12,13].

3. Results

3.1. Sequence analysis of the *B. japonicum phoB* gene

In the course of our attempts to identify alternative σ factors in *B. japonicum* we had cloned a 1.8-kb chromosomal fragment that hybridised to an *E. coli*

rpoS probe (data not shown). DNA sequence analysis of the insert of the resulting plasmid pRJ5510 revealed an open reading frame of 708 nucleotides. The corresponding gene product consists of 235 amino acids with a predicted molecular mass of 26895. A search in the GenEMBL data base using the program 'TFASTA' showed that the deduced amino acid sequence exhibited significant similarity to the phosphate regulatory protein PhoB of *S. meliloti* (71.4% identical amino acids; accession number M96261), *E. coli* (50.2%; P08402) and other bacterial species (Fig. 2). Moreover, the highly conserved phosphate-accepting residue Asp-53 as well as residue Thr-83, which plays an important role in the phosphate transfer as described for *E. coli* PhoB [14], are also present in the *B. japonicum* protein. On the basis of these similarities the predicted open reading frame found on pRJ5510 was designated *phoB*. As the cloned DNA fragment did not contain *rpoS*-like sequences, the reason for the initially observed cross-hybridisation with *E. coli rpoS* remains obscure.

3.2. Construction and phenotypic analysis of *phoB* mutants

B. japonicum phoB mutants 5518 and 5519 were constructed by insertion of a kanamycin resistance cassette into the *Nru*I site located in the *phoB* gene (see Fig. 1 and Section 2). The symbiotic properties of these mutants were tested in a plant infection test. The mutant strains did not differ from the wild-type

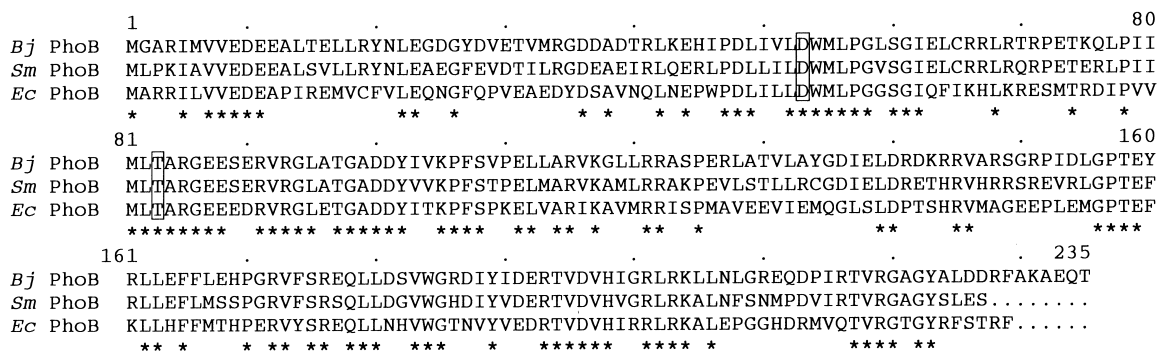


Fig. 2. Alignment of deduced amino acid sequences of the PhoB protein of *B. japonicum* (*Bj*, accession number AJ223073), *S. meliloti* (*Sm*, M96261), and *E. coli* (*Ec* [17]). Amino acids are given in the single-letter code. The asterisks indicate residues that are identical in all three sequences. The putative phosphate-accepting residue (D), and a second residue (T) that plays an important role in the phosphate transfer reaction of the *E. coli* protein, are boxed.

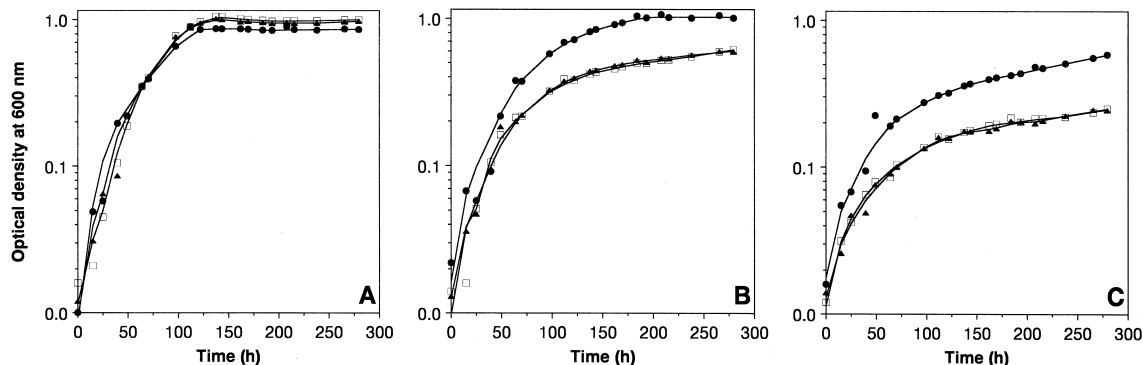


Fig. 3. Effect of phosphate-limited conditions on the growth of the *B. japonicum* wild-type and of *phoB* mutants. Cultures of *B. japonicum* wild-type (●) and *phoB* mutants 5518 (□) and 5519 (▲) were grown in minimal medium (HM) containing 88.1 μM (A), 26.4 μM (B), and 8.8 μM phosphate (C) under aerobic conditions at 28°C. The growth curves are based on mean values from two parallel cultures. Deviations between parallel cultures were less than 10%.

with respect to the ability to nodulate soybean roots and to fix nitrogen under symbiotic conditions. Nitrogenase activity was determined to be $108 \pm 9\%$ and $111 \pm 11\%$ of wild-type activity for *B. japonicum* 5518 and 5519, respectively. This indicates that the *phoB* gene product is not essential for symbiosis under the conditions tested.

The mutants were further analysed for their growth behaviour in media containing different phosphate concentrations. Aerobic growth of the mutants in rich medium (PSY medium) or minimal medium (HM medium) was not affected as compared with the wild-type (data not shown). Only when the phosphate concentration in the minimal medium was strongly reduced was growth of the mutants slowed down. Fig. 3 shows the behaviour of the wild-type and the mutants growing aerobically over a period of 280 h in minimal medium containing 88.1 μM , 26.4 μM , and 8.8 μM phosphate, which corresponds to 10%, 3%, and 1% of the phosphate in standard HM medium, respectively. At a phosphate concentration of 88.1 μM , the doubling times of the wild-type and the mutants were similar (19 h), and comparable final cell densities were reached (Fig. 3A), whereas at 26.4 μM phosphate the respective doubling times were calculated to be 21 h for the wild-type and 26 h for the mutants (Fig. 3B). After 280 h, the optical density of mutant cultures had reached only about half of that observed in wild-type cultures. This is due to a rather short exponential growth phase of the mutant cultures, as growth slowed down after

approximately 80 h. In the presence of 8.8 μM phosphate, limited growth was observed for both the wild-type and the mutant strains (Fig. 3C). Again the *phoB* mutants reached a lower final optical density after 280 h than the wild-type (0.25 and 0.58, respectively). Notably, no difference was observed between mutants 5518 and 5519 under all conditions tested.

4. Discussion

The identification of the *B. japonicum phoB* gene was based on the pronounced similarity of the deduced *phoB* gene product to PhoB proteins from other bacteria. Furthermore, a functional role of the *B. japonicum phoB* gene was documented by the impaired growth of *phoB* mutants under phosphate-limited conditions.

As shown for *E. coli*, a group of genes belonging to the *pho* regulon are activated by the regulatory protein PhoB during phosphorus limitation. The objective of this regulation is to achieve a higher phosphorus concentration in the cell by increasing the number of phosphate, organophosphate and phosphonate uptake and mobilisation systems [2,3]. The deletion of a phosphate uptake system can cause severe effects on cell growth as demonstrated by the mutant analysis of the *S. meliloti* PhoCDET system [4]. Mutants of *phoCDET* grew poorly at the relatively high phosphate concentration of 2 mM.

Assuming that *B. japonicum*, a rather close relative of *S. meliloti*, possesses a phosphate transport system functionally equivalent to PhoCDET, one may ask why the *B. japonicum phoB* mutants described here showed a growth defect only at a phosphate concentration lower than 25 μM . One possible explanation is that the phosphate transport system is synthesised at a basal level independently of PhoB. In fact, a basal expression of the high-affinity PhoB-regulated phosphate transport system PstSCAB (K_m 0.4 μM) was observed in *E. coli* under conditions of phosphate excess where PhoB is not active. However, under these conditions the majority of phosphate is taken up by the so-called Pit transporter, a low-affinity phosphate uptake system (K_m 38.2 μM) that appears to be synthesised constitutively (see [3], and references therein). A similar, alternative phosphate uptake system might also be present in *B. japonicum* which would enable the *phoB* mutants to grow normally at phosphate concentrations as low as approximately 100 μM . The presence of several phosphate uptake systems with different substrate affinities seems to be a common feature in bacteria because a *pit*-like gene has recently been identified also in *S. meliloti* [4,15].

Shortly before our work was submitted, Voegele et al. [15] published a thorough study on the PhoCDET high-affinity phosphate uptake system (K_m 0.2 μM) and the OrfA-Pit low-affinity system (K_m 1–2 μM) in *S. meliloti*. Under phosphate-limited conditions the PhoB regulatory protein activated the expression of the *phoCDET* operon and repressed the OrfA-Pit system. The failure of *S. meliloti phoCDET* mutants to grow under conditions of excess phosphate (2 mM) was apparently due to a reduced activity or expression of the OrfA-Pit system in these mutants via an unknown mechanism. By contrast, the normal growth of *B. japonicum phoB* mutants under high phosphate conditions would suggest that the function of an OrfA-Pit-like system, if it exists in this species, is not affected by the *phoB* mutation.

The growth phenotype of *B. japonicum phoB* mutants might explain why they showed no obvious defect in the plant infection test. The Jensen medium used to grow soybeans contains approximately 2.5 mM phosphate. Thus, it seems likely that bacteroids living in the root nodules are provided with non-limiting amounts of phosphate and, therefore, a de-

fective PhoB protein might cause no disadvantage for the cell in this assay. We considered it unrealistic to perform plant infection tests under phosphate limitation, because this condition affects not only *B. japonicum* but also soybean growth per se [16], which makes it difficult, if not impossible, to interpret in planta N_2 fixation data.

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References

- [1] Israel, D.W. (1987) Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiol.* 84, 835–840.
- [2] Torriani-Gorini, A., Yagil, E. and Silver, S. (1994) Phosphate in Microorganisms. Cellular and Molecular Biology. ASM Press, Washington, DC.
- [3] Wanner, B.L. (1996) Phosphorus assimilation and control of the phosphate regulon. In: *Escherichia coli and Salmonella*. Cellular and Molecular Biology (Neidhardt, F.C., Ed.), Vol. 1, pp. 1357–1381. ASM Press, Washington, DC.
- [4] Bardin, S., Dan, S., Osteras, M. and Finan, T.M. (1996) A phosphate transport system is required for symbiotic nitrogen fixation by *Rhizobium meliloti*. *J. Bacteriol.* 178, 4540–4547.
- [5] Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [6] Regensburger, B. and Hennecke, H. (1983) RNA polymerase from *Rhizobium japonicum*. *Arch. Microbiol.* 135, 103–109.
- [7] Cole, M.A. and Elkan, G.H. (1973) Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrob. Agents Chemother.* 4, 248–253.
- [8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Vols. 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [9] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [10] Ramseier, T. and Göttfert, M. (1991) Codon usage and G+C content in *Bradyrhizobium japonicum* genes are not uniform. *Arch. Microbiol.* 156, 270–276.
- [11] Alexeyev, M.F. (1995) Three kanamycin resistance gene cassettes with different polylinkers. *BioTechniques* 18, 52–54.
- [12] Hahn, M. and Hennecke, H. (1984) Localized mutagenesis in *Rhizobium japonicum*. *Mol. Gen. Genet.* 193, 46–52.

- [13] Göttfert, G., Hitz, S. and Hennecke, H. (1990) Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. *Mol. Plant-Microb. Interact.* 3, 308–316.
- [14] Makino, K., Amemura, M., Kim, S.-K., Nakata, K. and Shinagawa, H. (1994) Mechanism of transcriptional activation of the phosphate regulon in *Escherichia coli*. In: *Phosphate in Microorganisms. Cellular and Molecular Biology* (Torriani-Gorini, A., Yagil, E. and Silver, S., Eds.), pp. 5–12. ASM Press, Washington, DC.
- [15] Voegelé, R.T., Bardin, S. and Finan, T.M. (1997) Characterization of the *Rhizobium (Sinorhizobium) meliloti* high- and low-affinity phosphate uptake systems. *J. Bacteriol.* 179, 7226–7232.
- [16] Drevon, J.J. and Hartwig, U.A. (1997) Phosphorus deficiency increases the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta* 201, 463–469.
- [17] Makino, K., Shinagawa, H., Amemura, M. and Nakata, A. (1986) Nucleotide sequence of the *phoB* gene, the positive regulatory gene for the phosphate regulon of *Escherichia coli* K-12. *J. Mol. Biol.* 190, 37–44.